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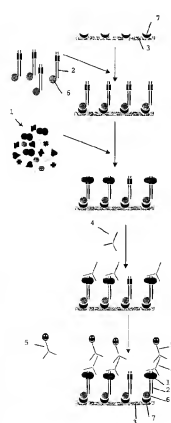
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(54) Title: METHOD AND KIT FOR THE SCREENING, THE DETECTION AND/OR THE QUANTIFICATION OF TRANSCRIPTIONAL FACTORS

(57) Abstract: The present invention is related to a screening, detection and/or quantification method of one or more transcriptional factor(s) (1) possibly present in a biological sample, said method comprising the steps of: possibly extracting and isolating said transcriptional factor (1) from said biological sample, putting into contact the transcriptional factor (1) with a double-stranded DNA sequence (2) bound to an insoluble solid support (3), and detecting and/or quantifying said fixed transcriptional factor (1), said double-stranded DNA sequence having a specific sequence able to be fixed by the transcriptional factor (1) and being preferably located at a distance of at least about 6,8 nm from the surface of the solid support (3), and said double-stranded DNA sequence being bound to the surface of the insoluble solid support (3) at a concentration of at least 0.01 pmole/cm<sup>2</sup> of solid support surface (3). The present invention is also related to the kit comprising means and media for performing said method.

WO 01/73115 A1



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METHOD AND KIT FOR THE SCREENING, THE DETECTION AND/OR THE  
QUANTIFICATION OF TRANSCRIPTIONAL FACTORS

10 Field of the invention

[0001] The present invention is related to a method and kit comprising means and media for the screening, the detection and/or the quantification of transcriptional factors or compounds binding said factors by non  
15 radioactive detection means.

Background of the invention

[0002] Transcriptional factors are proteins that bind to specific sequences of DNA, called consensus  
20 sequences, and influence the transcription of the DNA into mRNA. Some of these factors directly participate in the transcription process by activating or inhibiting the transcription and regulate the synthesis of proteins needed by cells to function, to adapt, to respond or to  
25 differentiate. Some of these proteins have to be transcribed in a constitutive manner (essential role in cell functions) while others are only synthesised in response to specific stimuli or when the cells are for instance in pathological environment. External signals are  
30 sensed by receptors and transduced through the plasma membrane followed by cascades of enzymatic kinase reactions, resulting in a phosphorylation or dephosphorylation of the transcriptional factors which

affects positively or negatively their binding to their consensus sequence.

- [0003] An example of drug acting indirectly on transcriptional factor is compactin, an inhibitor of HMG CoA reductase, which leads to an up-regulation of the transcription of the LDL receptor gene, permitting the clearance of cholesterol (US-A-5 563 036).

#### State of the art

- 10 [0004] There is a need for the detection and quantification of transcriptional factors (working as regulators and adaptators) for improving the diagnostic of pathologies or for developing drug affecting their activity.
- 15 [0005] Several methods are currently used to estimate the activation of transcriptional factors like NFkB. The most common method is to assay for their DNA-binding capacity by gel retardation, also called electrophoretic mobility shift assay (EMSA) (Schreck, R. et al, *Nucleic Acids Res*, 18(22), 6497-502 (1990)). This method is sensitive, but does not allow the simultaneously processing of numerous samples and requires particular precautions and equipments necessary for the handling of radioactivity.
- 20 [0006] A second largely used method is based on reporter genes (luciferase or  $\beta$ -galactosidase genes) placed under the control of a promoter containing the consensus sequence. This promoter can be artificial, made of several specific cis-elements and a TATA box, or natural, like the HIV long terminal repeat (LTR) element. However, other transcription factors influence the expression level of the reporter gene. In addition, as the read-out is the enzymatic activity of luciferase, for instance, the results may be affected by interferences with downstream processes

like general transcription or traduction machinery. This method is widely used provided the cells are efficiently transfected with the reporter plasmid.

[0007] Two other indirect methods are restricted to a few transcription factors. The first one uses antibodies raised specifically against the nuclear localising sequence (NLS) of transcriptional factors like NFkB, a part of the protein which is masked by IkB when the transcription factor is inactivated (Zabel, U. et al., *EMBO J.*, 12(1), 201-211 (1993)). The activation of NFkB can be visualised by immunofluorescence with this antibody. This method does not suit many samples analysis.

[0008] These various methods have been very helpful for fundamental research during these last 10 years, to unravel the molecular activation mechanisms of transcriptional factors like NFkB or AP1. Nevertheless, research has been hampered in this field by the fact that no convenient assay suitable for large scale screening procedure was available.

[0009] An assay for testing the inhibition by pharmacological agents of transcriptional factors binding to their specific sequence has been described in the patent US-A-5 563 036. The potentially active agent is incubated with the radioactively labelled transcriptional factor and the inhibition of its binding to the specific sequence is then assayed. This sequence is conjugated to biotin capable of specific binding to avidin immobilised onto microwells. A factor bound to the sequence and bearing biotin will be captured by avidin-coated plates and measured. The labelled protein, the nucleic acid conjugate and the compound form a mixture that is incubated with the avidin immobilised on the solid substrat . The binding of the factor to its

sequence is performed in solution for testing possible inhibition by a pharmaceutical agent present in solution.

[0010] In solution, the length of the nucleic acid probe ranges between 28 and 60 base pairs. This assay works  
5 with high concentration of factor in solution, but it is not at all sensitive for diagnostic assay in biological samples. Furthermore, probe excess has to be added in solution. The probe, which binds to avidin on the support, is preferably a free probe which has a smaller size and  
10 thus a much higher diffusion rate, so that the level of binding of probe/factor complex is low.

[0011] The patent US-5 747 253 describes a method for the identification of oligomers having a very high specific binding capacity for transcriptional factors.  
15 Synthesised oligonucleotides are first incubated with factors in solution in order to test their binding affinity to the factor, then assayed by attaching the oligonucleotide up to 25 bases to a support through a linker moiety present on the oligonucleotides. The reaction  
20 between the factor and the oligonucleotide is performed in solution, thereby limiting the sensitivity.

[0012] Magnetic microparticles can also be used to capture complexes formed between nucleic acids and proteins as proposed in document US-A-5 939 261. The reaction is  
25 performed in solution thus leading to the already mentioned limitations.

[0013] Gubler and Abarzua (Biotechniques, 18, 1008, 1011-4 (1995)) describe immobilised antibodies on the surface of wells to capture the transcriptional factor  
30 present in a solution. A biotinylated probe is added in the solution so that the active factors will be immobilised together with their probes that can then be detected with alkaline-phosphatase-streptavidin conjugate. However, all transcriptional factors either active or not will bind to

the antibodies. There will be a competition for the binding to the antibodies. Results on cell extracts showed a sensitivity of 2  $\mu\text{g}$  in the assay for the p53 protein.

[0014] Benotmane et al (Analytical Biochemistry 250, 185-185 (1997)) describe a protein-DNA binding of a recombinant protein at equilibrium, said recombinant protein, a fragment 6D3 of HLTF, having a DNA binding domain, the 6D3 portion of an helicase, linked to the glutathione S-transferase (GST). The binding coefficient and the dissociation coefficient in two experimental systems are determined by testing the binding of the biotinylated probe on a protein onto wells surface, or binding of the protein onto a consensus sequence of 27 base pairs fixed to the wells surface. In both cases they detect the protein-DNA binding. However they found a lower dissociation constant in the second test. There was no attempt to test the method on cell extract and it is difficult to assess the real sensitivity of the method given the fact that pure recombinant protein was used in this assay.

[0015] In cells or tissues, the transcriptional factors are present in minute amounts and this is the reason why the EMSA based on the incubation of the large excess of radioactive labelled probes in solution is the standard assay in the laboratories. In many cases, the concentration of the transcriptional factor is below the dissociation constant since the factors are diluted during the preparation of cell lysates. For this reason, an excess of probes allows the displacement of the binding reaction towards the formation of the DNA-protein complex. Secondly the low level of binding can be detected due to the high sensitivity of the  $\text{P}^{32}$  radioactive measurement. These difficulties explain also why alternative assays also use

radioactive labelling or are only valid when performed on purified proteins.

#### Aims of the invention

- 5 [0016] The present invention aims to provide a new and improved method and device for the screening, the detection (and possibly the quantification) of transcriptional factors or compounds binding said factors, preferably several of them being present in a biological  
10 sample, preferably by using non radioactive detection means.

#### Definition

- [0017] Transcriptional factors are proteins which  
15 bind to specific sequences of double stranded DNA, called consensus sequences, and when activated either by themselves or with the help of other proteins or enzymes will modulate, either activate or repress, the transcription of the DNA.
- 20 [0018] The transcriptional factors are usually found in both active or inactive forms, the shift from one form to the other being usually reversible. They are composed of a DNA binding domain and a transactivating domain responsible for their activity and they can be part of a  
25 large protein complex which interacts with the transcriptional machinery for regulating its activity.
- [0019] The following method according to the invention may also be used for the screening, detection and/or quantification of proteins which bind to specific  
30 double stranded DNA. One such protein is the HIV integrase which recognises the sequence 5'-GTGTGGAAAATCTCTAGCA-3' with a possible GT at the 3' end which is cut by the enzyme. The enzyme can be stabilised in its binding form using specific experimental conditions mainly the presence



of  $Me^{++}$  ( Yi et al. Biochemistry 38, 8458, 1999). Other viral proteins binding to DNA sequence are listed in table 1 and are also possibly detected by the present invention.

5

#### Summary of the invention

[0020] The present invention is related to a screening and/or quantification method of one or more transcriptional factor(s) 1 present in a cell or a cell  
10 lysate (including nuclear lysate of a cell), said method described in the enclosed figure 1 comprising the steps of:

- binding to an insoluble solid support 3, double-stranded DNA sequence(s) 2 at a concentration of at least about 0.01 pmole/cm<sup>2</sup>, preferably 0,1 pmole/cm<sup>2</sup>, of said solid  
15 support surface (step 1), said double-stranded DNA sequence 2 further comprising a specific nucleotide sequence able to bind specifically said transcriptional factor(s) 1,
- putting into contact said transcriptional factor(s) 1  
20 with said bound double-stranded DNA sequence(s) 2, and
- identifying and/or quantifying a signal resulting from the binding of said transcriptional factor(s) 1 to said double-stranded DNA sequence(s) 2.

[0021] Preferably, said signal is obtained by the  
25 following step:

- after washing, the presence of the fixed transcriptional factor 1 is detected by using firstly a primary antibody 4 raised against the factor 1 (step 2) and then a secondary labelled antibody 5 (for example  
30 conjugated with an enzyme such as peroxidase) directed against the primary antibody 4 (step 3).

[0022] The presence of the peroxidase is then advantageously assayed through the addition of a suitable

substrate by a colorimetric detection of the reaction product in solution (step 4). Such non-radioactive method is rapid, more sensitive than gel retardation, and suits for large-scale screening or detection upon various solid supports.

[0023] It is meant by "a specific nucleotide sequence of a double-stranded DNA sequence able to bind a transcriptional factor", a nucleotide sequence which can be recognised specifically through biochemical interactions by said transcriptional factor(s).

[0024] The present invention is sensitive enough to be applied for the detection of transcriptional factors or proteins able to bind DNA sequence present in cell lysate (including nuclear lysate) or present in a body fluid. Unexpectedly, less than about  $10^{-12}$  mole of transcriptional factor can be detected in 50 micro-litres in an assay using the method of the present invention and the limit of detection was found to be of about  $5 \cdot 10^{-16}$  mole for NF-KB assay. This sensitivity explains the possibility of using such invention for the detection of transcriptional factors naturally present in cell lysate. The sensitivity obtained which is at least as good as the EMSA method (example 2), is a surprising finding since the DNA consensus is not in solution, but fixed on (bound to) a solid surface (where the reaction is slower than in solution) and the detection does not require the radioactive measurement but may be obtained by simple colorimetry.

[0025] Preferably, in the method according to the invention, the specific nucleotide sequence of the double-stranded DNA sequence(s) able to bind with the transcriptional factor(s) is located at a distance of at least 6.8 nm from the surface of the solid support.

[0026] According to a preferred embodiment of the present invention, the double-stranded DNA sequence may

comprise a spacer of at least about 13.5 nm between the specific sequence and the surface of the solid support.

[0027] Preferably, said spacer is a double-stranded DNA nucleotide sequence of at least 20 base pairs, 5 preferably at least 50, 100, 150 or 250 base pairs.

[0028] Another aspect of the present invention is related to a screening, diagnostic and/or quantification kit comprising means and media for performing the method according to the invention.

10 [0029] Preferably, the invention is related also to a screening and/or quantification kit or device including high throughput screening device, possibly comprising computer-controllable electromagnetic means and robots (such as high-throughput screening device) allowing the 15 screening and detection upon any type of solid support 1 and used for the screening and/or quantification of transcriptional factor(s) (or compounds able to bind said transcriptional factor(s) or inhibit the binding of said transcriptional factor) to said specific nucleotide 20 sequence comprised in the double-stranded DNA bound to the insoluble solid support at a concentration of at least about 0.01 pmole/cm<sup>2</sup> of solid support surface; said specific nucleotide sequence being located at a distance of at least about 6.8 nm from the surface of the solid 25 support.

[0030] The screening and/or quantification kit according to the invention comprises also means and media for detecting the signal resulting from the binding of the transcriptional factor(s) to the double-stranded DNA 30 sequence(s), said signal being a non-radioactive resulting signal.

[0031] The method and kit according to the invention is also suitable for the (possibly simultaneous) screening

and/or quantification of multiple different transcriptional factors present in a same biological sample (cell or cell lysate). The method is especially well adapted for the detection of multiple transcriptional factors, on a same support (one biochips or one multiwell plate). However, the detection of the different factors from a same sample can also be performed in wells of different plates.

[0032] The method according to the invention may comprise also the step of screening and/or quantifying compound(s) able to bind to said transcriptional factor(s) (or inhibit their binding) to the specific sequence, preferably by using steps, means and media such as the ones described in the US patent 5,563,036.

[0033] The method according to the invention may also comprise the step of identification of at least one characteristic specific of a given transcriptional factor activation. Some transcriptional factors can bind to their consensus DNA sequence without activating the transcriptional machinery. The activation is then associated with one or several specific changes, the most common one being the phosphorylation (or dephosphorylation) at specific amino acid(s) of the protein. An example of such transcriptional factor is CREB, which is only active when phosphorylated (example 1).

[0034] The method according to the invention may also comprise the step of screening and/or quantifying known and unknown compound(s) able to modulate the activation of said transcriptional factor(s) in cells, tissues or organisms and detecting the residual activity of the transcriptional factor(s) in cell lysate. External protein having at least in part the binding DNA domain can also be added to the incubation solution in order to determine its possible activation by cell lysate and to obtain a screening of compounds acting upon this

activation. If a purified transcriptional factor is added to the cell lysate, its activation can be quantified by determination of one or several of its specific characteristics. Proteins acting as activators or  
5 inhibitors upon a transcriptional factor activation can also be added in the incubation medium and serve in a screening method of compounds acting upon these proteins.

[0035] The method may also comprise the step of forming a mixture by combining a labelled protein  
10 comprising a portion of the transcriptional factor with the specific nucleotide sequence being bound to the insoluble solid support. Thereafter, the method comprises the step of incubating said mixture in the conditions, whereby in the absence of the compound, the labelled protein binds to the  
15 nucleotide sequence, the step of separating from the solid support a fraction of said mixture, whereby the fraction comprises the labelled protein if said labelled protein is not bound to the sequence and the step of detecting the presence or absence of said labelled protein upon said  
20 solid support; wherein the absence of said detected label upon said solid support, means that the compound inhibits the binding of a transcriptional factor to the nucleotide sequence. Preferably, said method comprises also the step of recovering the detected unknown compound.

25 [0036] According to the invention, the solid support is preferably an array bearing at least 4, 10, 16, 25, 100, 1000, 10.000 or more spot/cm<sup>2</sup> of solid support surface, each spot containing at a specific location double-stranded DNA sequence(s) for the binding of transcriptional  
30 factor(s).

[0037] According to a preferred embodiment of the present invention, the double-stranded DNA sequence 2 is bound to a first member 6 of a binding pair such as

biotin/streptavidin, hapten/receptor, antigen/antibody), able to interact with the second member 7 of said binding pair, bound to the surface of the solid support 3.

[0038] Alternatively, the double-stranded DNA  
5 sequence(s) could be covalently bound to the surface of the insoluble solid support.

[0039] Alternatively, the method according to the invention could comprise also the step of identification of transcriptional factor(s) and/or protein(s) which are part  
10 of their active complex.

[0040] The method according to the invention may also comprise the step of screening or detecting the "fixed" transcriptional factor(s) and possibly quantifying the presence of said "fixed" transcriptional factor(s)  
15 (and/or possibly quantifying the concentration of the transcriptional factor(s) present in a biological sample (cell or cell lysate)).

[0041] The use of a consensus DNA sequence 2 already fixed on a solid support 3 allows the industrial production  
20 with reproducible and quality control of the kit or device containing such prepared support. It also simplifies the use of the kit since a biological sample (cell or cell lysate) can be incubated directly upon such prepared solid support surface without requiring the addition of any other  
25 reagent.

[0042] Said general method for detecting the DNA-binding capacity of transcriptional factors is enough sensitive, specific and valid for most (if not all) transcription factors. Unexpectedly, it is possible to  
30 improve said sensitive method, by increasing the size of the spacer between a consensus sequence on which the transcriptional factor will bind and by using a very high density of these nucleotide sequences attached on the solid support surface. The method is so sensitive that it can be

used for the detection of one factor present in a biological sample, but also for the detection of several factors by the use of multiple nucleotide sequences attached in distinctive spots on a solid surface such as  
5 biochips (microarrays).

[0043] The minimum spacer was found to be a nucleotide sequence, such as a DNA sequence, of 20 base pairs (bp), but said spacer may have between 50 and 250 base pairs according to the type of solid support and  
10 antibody used (Example 4). The presence of the consensus nucleotide sequence at long distance from the solid support surface can be obtained similarly through the presence of a chemical spacer of at least 10 atoms, preferably of at least 44 atoms, more preferably of at least 50 atoms or  
15 more.

[0044] Said method, kit or device are suitable for testing the activity of pharmaceutical drugs or methods acting upon these transcriptional factors (i.e. as inhibitors and/or activators of their binding to the DNA)  
20 or on their regulatory processes: activation or repression.

[0045] The invention is also related to said compound identified or recovered and possibly integrated in a pharmaceutical composition for preventing or treating various symptoms or diseases.

25 [0046] Said screening, detection and/or quantification method and kit are suitable for detection and quantification of all the transcriptional factors for which the DNA binding sequence is known. These transcriptional factors and their properties are listed in  
30 the Web site "<http://transfac.gbf.de/TRANSFAC/cl/cl.html>"  
The most commonly tested factors are selected from the group consisting of NF-KB, AP-1, CREB, SP-1, C/EBP, GR, HIF-1, Myc, NF-AT, Oct, TBP and CBF-1. A list of some

transcriptional factors and their corresponding consensus (sequences SEQ ID No. 1 to 125) is given in table 1.

[0047] NF $\kappa$ B is an important ubiquitous transcriptional factor activated following cell stimulation, involved in the immune response to some viral and bacterial products, oxidative stresses or pro-inflammatory cytokines (Baeuerle, P. A. and Baichwal, V. R., *Adv Immunol*, 65, 111-37 (1997)).

[0048] AP1 is also dimeric transcriptional factor which is involved in many cell responses through activation of kinase cascade. This transcription factor is involved in a variety of biological processes like cell growth, differentiation, or apoptosis.

[0049] Preferably, said detection and possibly quantification is obtained by the use of compounds that are able to specifically bind to the transcriptional factor fixed upon the double-stranded DNA, such as (preferably monoclonal) antibodies or specific hypervariable portions thereof (Fab', Fab2', etc.).

[0050] According to a preferred embodiment of the present invention, said binding is followed by incubation (and washing) with labelled compounds able to react with the first compound binding to said transcriptional factor, preferably (monoclonal) antibodies directed against the anti-transcriptional factor antibodies or specific hypervariable portions thereof (Fab', Fab2', etc.). Said last antibodies are preferably labelled with non radioactive markers allowing a detection, preferably by colorimetry, fluorescence, bioluminescence, electroluminescence or precipitation of a metal deposit (such as silver staining) as in example 3. Other secondary binding proteins like protein A which bind to antibodies are also an embodiment of the method. Said non radioactive test is preferably based upon a colorimetric assay



resulting from an enzymatic activity such as described in the enclosed Fig. 1. If direct method such as mass spectrum analysis is sensitive enough it can be used for direct detection of the bound factor.

- 5 [0051] The selected antibody recognises an epitope which is accessible when the factor is in its active form and bound to DNA. Usually, the active and inactive forms of the factors differ in their phosphorylation state or by the presence of an inactivator protein. The antibody has to  
10 recognize the active form. The concentration of these transcription factors in a cell is very low and may reach the limit of the affinity coefficient of the antibodies. An antibody with a high affinity (i.e. with very low coefficient of dissociation) can highly increase the signal  
15 obtained. However, any other protein which has an affinity for the factor can be used for the detection.

- [0052] Special applications are the use of specific antibodies directed against phospho-protein epitopes or against specific proteins which are part or can be attached  
20 to the transcriptional factor (see example 1). For NF- $\kappa$ B, antibodies directed either against P-50 or P-65 allow to determine the pattern of association of proteins giving the NF- $\kappa$ B activity.

- [0053] The present invention also allows a  
25 differentiation between the DNA binding of the transcription factor and its activity. This activity is associated with one or several particular characteristics of the factors, the most common one being the phosphorylation at specific locations or the dissociation  
30 of inhibitory proteins. By using appropriate antibodies directed against these elements, it is possible to determine the amount of transcriptional factors in their active form. As an example, CREB can bind to its DNA

consensus sequence without being active. Its activation results from a specific phosphorylation at a serine group, making the P-CREB. The present invention detects both the CREB binding and the P-CREB activation by using antibodies  
5 directed either against CREB or specific of the P-CREB (example 1).

[0054] The attached transcriptional factor can also be used to test for the presence of proteins which have affinity for the transcriptional factor.

10 [0055] The method comprises also the step of screening and/or quantifying known and unknown compound(s) able to modulate the activation of said transcriptional factor by modification of the level of specific characteristics of said factor.

15 [0056] If the binding is specific and if no other proteins are used on the nucleotide sequence binding on the surface, for example when the nucleotide sequences are directly linked to said surface, then a direct detection of protein will give an estimation and/or quantification of  
20 the bound factor.

[0057] A signal obtained in each well or for each spot of a biochip is recorded and the means of the signal are calculated for each identical consensus sequence. Usually, two (and preferably three to five) identical spots  
25 are present on each array in order to correct variations that may occur at any step of the process. The background values are wells or spots coated with a consensus sequence, in which binding buffer and lysis buffer is added instead of a cell extract or purified transcription factor  
30 (negative control). A positive control is preferably added as a factor obtained in a pure active form and for which a consensus nucleotide sequence is attached to the surface of the solid support in the test.

[0058] Quantification has to take into account not only for the binding yield, but also the detection part of the process and the reading scale. Internal or external standards using purified active transcriptional factor can  
5 be added to the sample at either step of the process in given amount as reference value to which the results will be compared. Validation of the use of these standards has to be performed earlier in order to validate their binding efficiency compared to the tested factors.

10 [0059] In the case of biological applications, comparison of transcriptional factors involved in the cell response can be quantified in comparison to transcriptional factors which are constitutively active (there are factors which regulate the expression of house keeping genes). The  
15 use of these "relative" quantification simplifies the assays, since all factors are treated in the same way during the various steps.

[0060] The simultaneous detection of several transcriptional factors binding or activity is especially  
20 usefull since it gives a pattern of response of the cells or tissues to a particular biological situation, to molecules or drug action. The transcriptional factors are activated by complex pathways which are related to each other. A general screening analysis of different  
25 transcriptional factors will give a better view and help the interpretation of the studied effects.

[0061] Furthermore, solid supports such as a biochips can be inserted in a machine support connected to another chamber and automatic machine through the control  
30 of liquid solution based upon the use of microfluidic technology. By being inserted into such a microlaboratory system, it can be incubated, heated, washed and labelled by automates, even for previous steps (like a PCR amplification) or the following step (labelling and

detection). All these steps can be performed upon the same solid support.

[0062] According to the invention, the solid support is preferably selected from the group consisting of glass, 5 metallic supports, polymeric supports (preferably a polystyrene support activated with carboxyl or amino groups present on its surface) or any other support used in the microchips (or micro-arrays) technology (preferably activated glass bearing aldehyde groups), said support 10 comprising also specific coatings, markers or devices (bar codes, electronic devices,...) for improving the assay.

[0063] If glass presents many advantages (like being inert and having a low auto-fluorescence), other supports like polymers, with various chemically well-defined groups 15 at their surface, allowing the binding of the nucleotide sequences are useful.

[0064] Miniaturisation allows to perform one assay onto a surface (usually circular spots of about 0.1 to about 1 mm diameter). A low density array, containing 20 to 20 400 spots is easily obtained with pins of 0.25 mm at low cost. Higher density of spots going to 1,600 spots per cm<sup>2</sup> can be obtained by reducing the size of the spots for example to 0.15mm. The advantages of this technology is the high number of data which can be obtained and analysed 25 simultaneously, the possibility to perform replicates and the small amount of biological sample necessary for the assay.

[0065] The arrays technology allows the simultaneous detection of different factors present in the same sample, 30 which is possible due to the fact that several factors bind to their consensus sequence in the same reacting conditions. Solutions are available for example from Promega (Madison, Wisconsin, USA) for the binding of several factors (Catalogue E3581). Conditions which allow

the binding of the factors studied may be optimised by the person skilled in the art. Other binding assays involving DNA-DNA recognition, antigen-antibody or receptor-ligand recognition can be performed simultaneously on the same  
5 chips for other molecules.

[0066] The detection of the specific binding between the transcriptional factor and the double-stranded DNA sequence is obtained by any one of the methods described previously, or preferably by a method allowing a  
10 precipitation of a metal deposit (such as silver staining) at the location where the transcriptional factor has fixed (recognised) the double-stranded DNA sequence).

[0067] As only activated transcriptional factors are captured by the nucleotide sequences bound to the solid  
15 support (preferably microwell plates or microarray), the design of these sequences and binding conditions allow a very high sensitivity necessary for assays in biological samples.

[0068] The present invention will be described in  
20 more details in the following non limited examples in reference to the enclosed drawings.

#### Short description of the drawings

[0069] Figure 1 is a schematic presentation of the  
25 procedure for an assay of a transcriptional factor according to the invention.

[0070] Figure 2 represents a DNA-binding assay of 3 different transcription factors (NFkB, CREB and AP-1) measured simultaneously in the same cell extracts in  
30 microwells. Microwells contain a 100 bp DNA terminated by the sequence specific for either NFkB, CREB or AP-1, and the DNA-binding capacity of the transcription is detected respectively with anti-p65, anti phospho-CREB and anti-c-

fos (for AP-1 assay). The DNA-binding assay is performed on cell lysates coming either from cells left unstimulated, or stimulated with IL-1, with forskolin, with PMA+ionomycin, with IL-1 and forskolin, with IL-1 and PMA+ionomycin, with  
5 forskolin and PMA+ionomycin, and IL-1 and forskolin and PMA+ionomycin. The results show the signal obtained for the assay of NF-KB (A), P-CREB (B) and c-fos (C) on the various cell extracts (NF-KB is strongly induced in IL-1 stimulated cells, P-CREB by forskolin stimulation and AP-1 by PMA).

10 [0071] Figure 3 presents the sensitivity of the DNA-binding assay. DNA-binding activity of purified NFkB (p50) was measured on microwells containing DNA probe specific for NFkB. The concentrations of p50 used in this assay range from  $10^{-12}$  mole to  $5 \cdot 10^{-16}$  mole/well.

15 [0072] Figure 4 presents the sensitivity of the DNA-binding assay. DNA-binding activity of stimulated cells and unstimulated cells for NFkB.

[0073] Figure 5 presents the values obtained for the detection of NFkB present in cells stimulated or not with  
20 IL-1 on microarray by using DNA probes of different lengths containing a spotted consensus sequence for NFkB

[0074] Figure 6 presents the effect of various drugs on the DNA-binding activity of IL-1-induced NFkB.

## 25 Examples

### Example 1: Microwell colorimetric DNA-binding assay. (figures 1 and 2)

#### Step 1 : Binding of the double strand oligonucleotidic probe on multi-well plates

30 [0075] The spacer double strand nucleotide sequences were constructed from the following CMV sequence:

5'TGGCCAAGCGGCCTCTGATAACCAAGCCTGAGGTTATCAGTGTAATGAAGCGCCGCA  
TTGAGGAGATCTGCATGAAGTCTTTGCCCCAGTACATTCTGGGGGCCGATCCTCTGAGA

GTCTGCTCTCCTAGTGTGGATGACCTACGGGCCATCGCCGAGGAGTCAGATGAGGAAGA  
 GGCTATTGTAGCCTACACTTTGGCCACCGCTGGTGTGAGCTCCTCTGATTCTCTGGTGT  
 CACCCCCAGAGTCCCCTGTAC acting as a spacer was linked to a)  
 the NFkB consensus oligonucleotide  
 5 'AGTTGAGGGGACTT'TCCAGGC-3' b) the CREB consensus  
 oligonucleotide 5'ATTGCTGACGTCAGAGAGCTAG-3'  
 and c) the AP-1 consensus oligonucleotide 5'  
 CCGTTCGGCTGACTCATCAAGCG-3'. In the example the spacer was  
 of 100 based pairs. The CMV extremity is 5' biotinylated,  
 so that these probes can be linked to streptavidin-coated  
 96-wells plates : 2 pmoles of probes per well are incubated  
 1h at 37 °C in 50 µl 10 mM phosphate buffer 150 mM NaCl  
 (hereafter called PBS<sub>150</sub>). Plates are then washed and the  
 amount of DNA fixed on streptavidin-coated plates was  
 quantified using the picogreen assay (Molecular Probes, OR,  
 USA). One picomole of DNA was found to be fixed on the wells  
 using DNA standard for calibration of the assay.

## Step 2 : Binding of the transcription factor on the double strand probe

[0076] This assay was either performed with purified  
 p50-p50 (Promega, Madison, WI, USA), or with nuclear cell  
 extracts from SV-40 transformed WI-38 fibroblasts (WI-38  
 VA13 cell line). Cells were purchased from the ATCC and  
 plated in 75 cm<sup>2</sup> flasks. They were serially cultivated in  
 Minimum Essential Medium (Gibco, UK) supplemented with  
 Na<sub>2</sub>SeO<sub>3</sub> 10<sup>-7</sup> M and antibiotics, in the presence of 10 %  
 fetal bovine serum. Cells were stimulated for 30 min with  
 IL-1 (to activate NFkB), forskolin (to activate CREB),  
 PMA+ionomycin (to activate AP-1) or left unstimulated.  
 The nuclear cell extracts were prepared as followed  
 - cells were rinsed with cold PBS<sub>150</sub>

- cells were left swollen during 3 min in a cold hypotonic buffer (20 mM Hepes pH 7.4, 5 mM NaF, 1 mM  $\text{Na}_2\text{MoO}_4$ , 0.1 mM EDTA).
  - Cells were scrapped in 0.5 ml of hypotonic buffer
  - 5 containing 0.2 % nonidet P40 and immediately centrifuged during 30 sec at maximal speed (13000 rpm).
  - The pellet was resuspended in 100  $\mu\text{l}$  of lysis buffer (hypotonic buffer containing 20 % glycerol, 0.4 M NaCl, phosphatases and proteases inhibitors cocktail).
  - 10 - The nuclear extract was agitated 30 min at 4°C, and then centrifuged for 10 min at maximal speed (13000 rpm) at 4°C.
  - The supernatant ( = nuclear extract) was frozen at -80°C and its proteic content was determined by the Bradford
  - 15 assay.
- [0077] 20  $\mu\text{l}$  of p50 diluted in lysis buffer or 20  $\mu\text{l}$  of nuclear cell extract are incubated with 30  $\mu\text{l}$  of binding buffer (AAT, Namur) in microwells coated either with the consensus oligonucleotide for NF $\kappa$ B, or with the consensus
- 20 oligonucleotide for CREB, or with the consensus oligonucleotide for AP-1. After 2h incubation at room temperature with a mild agitation (200 rpm on IKA MS2 vortex, Germany), microwells are washed 3 times with PBS<sub>150</sub>. with 10 mM phosphate buffer 50 mM NaCl (hereafter called
- 25 PBS<sub>50</sub>) + Tween 0.1%, and once with PBS<sub>50</sub> alone.

### Step 3 : Binding of anti-NF $\kappa$ B on the NF $\kappa$ B-DNA complex

- [0078] 100  $\mu\text{L}$  of rabbit antibodies diluted 500 or 1000 times in 10 mM phosphate buffer 50 mM NaCl (hereafter
- 30 called PBS<sub>50</sub>) and 1% bovine serum albumine are incubated in each well for 1h at room temperature. The different antibodies used are :



- for NFkB : anti p65 (Santa Cruz #sc-372) or anti p50 (Santa Cruz #sc-7178)
- for CREB : anti CREB (Rockland, #100-401-195) or anti phospho-CREB (Upstate Biotechnology 06-519).
- 5 - for AP-1 : anti c-fos (Santa Cruz, #sc-7202) or anti c-jun (Biolabs #9164).

[0079] The microwells are then washed 3 times with PBS<sub>150</sub> + 0.1 % Tween.

10 Step 4 : Binding of peroxidase-conjugated anti-rabbit IgG

[0080] 100 µL of diluted peroxidase-conjugated anti-rabbit IgG (#611-1302, Rockland, Gilbertsville, PA, USA) diluted 1000 times in PBS<sub>50</sub> and 1% non fat dried milk are incubated in each well for 1h at room temperature.

- 15 Microwells are then washed with 200 µl PBS<sub>50</sub> + 0.1 % Tween.

Step 5 : Colorimetric revelation

[0081] 100 µl of tetramethylbenzidine (Biosource, Belgium) are incubated 10 min at room temperature before  
20 adding 100 µl of stopping solution (Biosource, Belgium). Optical density is then read at 450 or 405 nm, with an Ultramark microplate reader (Biorad, CA, USA).

- [0082] This procedure can be followed to quantitate the DNA-binding activity of different transcription factors  
25 in the same nuclear extract. In figure 2, the DNA binding activity of NFkB (a), CREB (b) and AP-1 (c) has been measured on the same samples following the procedure described here above. The samples are nuclear extracts of WI-38 VA13 cells left unstimulated or stimulated during 30  
30 min with 5 ng/ml of IL-1 (a NFkB activator), 10 µg/ml of forskolin (a CREB activator), 0.1 µg/ml PMA + 1µM ionomycin (a cocktail known to activate AP-1), or the different combinations of these activators. The results are

expressed as the percentage of the signal obtained with the regular activator the transcription factor assayed (for example, the NFkB DNA-binding activity is expressed as the percentage of the NFkB DNA-binding activity measured in 5 cells stimulated with IL-1 alone).

[0083] The results clearly show that the colorimetric DNA-binding assay allows to measure simultaneously the DNA-binding activity of several transcription factors in the same biological sample.

10

Example 2 : Sensitivity of the DNA-binding colorimetric assay in multiwells (figures 3 and 4)

[0084] To estimate the sensitivity of the colorimetric DNA-binding assay described in example 1, the 15 assay was performed with the NFkB specific probe first on various quantities of purified p50 (figure 3) and then on different quantities of nuclear cell lysates (figure 4).

[0085] Different quantities of purified p50 ranging from  $10^{-13}$  M to  $5.10^{-16}$  M were incubated in the microwells 20 containing the NFkB specific probe, and the assay was realised as described in example 1, using anti-p50 antibody. The results presented in figure 3 clearly show that this assay is extremely sensitive as the DNA-binding activity of as low as  $5.10^{-16}$  mole/well of purified p50 can 25 be detected.

[0086] The sensitivity of the colorimetric DNA-binding assay with cell lysates is also very high, as shown in figure 3. Cell extracts are coming either from unstimulated cells or from cells stimulated with IL-1 in 30 order to proceed for the assay as described in the example 1. In this experiment the concentrations ranged between 0.5 and 50  $\mu$ g per well. The assay was performed in parallel with EMSA, on the same protein extracts. The results

presented on figure 4 clearly show that the NF $\kappa$ B DNA-binding assay in microwells is more than 10 times more sensitive than the EMSA : 5  $\mu$ g of proteins were required to detect a first detection signal by EMSA, but less than 0.5  $\mu$ g of proteins was necessary for the microwell assay.

Example 3: Microarray assay for DNA-binding activity of NF- $\kappa$ B CREB in biological samples

Step 1 : Binding of the probes on glass

10 [0087] Activated glass slides bearing aldehyde groups were purchased from AAT (Belgium).

[0088] The slides were first incubated in 0.8 ml of a 50  $\mu$ g/ml streptavidin solution in a 10 mM phosphate buffer (pH 7.4) containing 10 mM NaCl (PBS<sub>10</sub> buffer). After  
15 1h incubation at 20°C, the plates were washed 3 times for 2 min in PBS<sub>150</sub> containing 0.02% Tween 20 and then 2 times 2 min with water. The plates were then incubated for 2h at 20°C in 20 ml of PBS<sub>150</sub> solution containing 10 % non fat dried milk and then washed 5 times 2 min in PBS<sub>150</sub>  
20 solution.

[0089] Spotting was performed with the NF $\kappa$ B consensus probe or with the CREB consensus probe as in example 1. The capture probes were printed onto the microscopic slides with a home made robotic device. 250  $\mu$ m  
25 pins from Genetix (UK) were used. The spots have an average of 400  $\mu$ m in diameter and the volume dispensed is about 1 nl. The concentration of probes used was 3000 nmolaire. Slides were incubated for 1h at room temperature, washed twice for 2 min with PBS<sub>50</sub> containing 0.02% Tween 20  
30 and then 3 times for 2 min in water.

Step 2 :Binding of transcription factor on the double strand probe fixed on micro-array

[0090] This assay was performed with nuclear extracts from WI-38 VA 13 fibroblasts stimulated with IL-1 $\beta$  or left unstimulated. The cell extracts were prepared as described in the example 1. The negative control was 20  $\mu$ l of lysis buffer mixed with 30  $\mu$ l of binding buffer. 20  $\mu$ l of p50 diluted in lysis buffer or 20  $\mu$ l of nuclear cell extract are incubated with 30  $\mu$ l of binding buffer (AAT, Namur, Belgium) per incubation chamber. After 1h incubation at room temperature with a mild agitation (200 rpm on IKA MS2 vortex, Germany), the arrays were washed 3 times.

Steps 3, 4 and 5 : Binding of anti-NF $\kappa$ B on the NF $\kappa$ B-DNA complex and detection

[0091] The binding of the primary anti-NF $\kappa$ B (anti-p50) antibody was then used for reaction on the factor followed by a washing and an incubation with a secondary antibody. In this case, the second antibody was gold-labelled. After 3 incubations of 10 min in a mixture of silver enhancer A, the slides were washed with water.

[0092] There was no signal for the negative control, while in nuclear cell extracts, activated NF $\kappa$ B is detectable in cell stimulated with IL-1, with a much higher intensity than in unstimulated. The results obtained with the nuclear cell extracts have been quantitated, using a colorimetric microarray reader including the quantification software (AAT, Namur, Belgium). The intensity of each spot was estimated by averaging the value of all pixels inside its boundaries. The value of the mean background around the spot was subtracted from the spot values. The value for the unstimulated cells was of 10 and the one for the stimulated cell were of 175 grey intensity.

[0093] As an alternative fixation of the probes, the consensus sequences were aminated by using a 5' terminal

aminated primer and amplified by PCR before being spotted directly on the aldehyde glass slides. The detection method was then processed in the same way as here above.

5 Example 4 : Influence of the spacer on microarray assay of NFkB and CREB assay on biological samples (figure 5)

Step 1 : Binding of the probes on glass.

[0094] The binding of probes was obtained as described in the example 3 using the consensus sequence of  
10 NF-KB or CREB alone or with a spacer of 50, 100, 150 and 250 bp constructed from the sequence given in example 1.

Step 2 : Binding of NFkB on the double strand probe fixed on micro-array

15 [0095] This assay was performed with whole cell lysates from WI-38 VA 13 fibroblasts stimulated with IL-1 $\beta$  (a NFkB activator) or 10  $\mu$ g/ml of forskolin (to activate CREB). The control was 20  $\mu$ l of lysis buffer mixed with 30  $\mu$ l of binding buffer.

20

Steps 3, 4 and 5 : Binding of antibodies and detection

[0096] The binding of the primary anti-NFkB and anti-PCREB antibodies was then used for reaction on the factors followed by a second gold labelled antibody and a  
25 colorimetric detection as in example 3. The results with increasing spacer length for the NF-KB is shown in figure 5. The figure shows the total length of the fixed DNA probes. The values for P-CREB with unstimulated cells were 5.5 and 4 respectively with no spacer and with a 100 base  
30 spacer. The values for stimulated cells were respectively of 7 and 21 respectively with no spacer and a spacer of 100 bases pairs.

Example 5 : Use of the DNA-binding colorimetric assay to screen for DNA-binding interacting drugs (figure 6)

[0097] The DNA-binding colorimetric assay was used for screening of molecules, like drugs, that can interfere either directly with the DNA-binding capacity of a transcription factor or with the upstream cellular activation process of a transcription factor.

[0098] In figure 6, several molecules known as drugs were screened for their eventual inhibitory effect on the DNA-binding capacity of 3 transcription factors. 5  $\mu$ g of nuclear extracts prepared as in example 1 from a) IL-1-stimulated fibroblasts or b) Forskolin-stimulated cells or c) PMA+ionomycin-stimulated cells were assayed respectively for the DNA-binding capacity of NFkB, CREB and AP-1, in the presence or absence of various neurotropic drugs at  $10^{-4}$ M.

[0099] The molecules were tested on the activation of the factors in the following way. Cells were incubated in the presence of the molecules at a concentration of  $10^{-5}$ M for one hour before the stimulation with IL-1, Forskolin or PMA+ionomycin. Nuclear extracts were prepared and the activity of the transcriptional factors measured as described in example 1.

Table 1 :List of transcriptional factors and their consensus sequences

5	AAF	TTTCATATTACTCT (SEQ ID NO:1)
	AbdB	AA(A/T)TTTTATTAC (SEQ ID NO:2)
	AhR	TGCGTGAGAAGA (SEQ ID NO:3)
	Antp	AA(A/T)TTTAATTAC (SEQ ID NO:4)
	Ap1	TGASTMA (SEQ ID NO:5)
10	AP2	CCCMCNSSS (SEQ ID NO:6)
	AP3	TGTGGWWW (SEQ ID NO:7)
	AP4	YCAGCTGYGG (SEQ ID NO:8)
	AR	AGAACANNNTGTTCT (SEQ ID NO:9)
	ARNt	GTG (3'-half site) (SEQ ID NO:10)
15	ARP-1	TGANCCCTTGACCCCT (SEQ ID NO:11)
	ATF	TGACGYMR (SEQ ID NO:12)
	BGP1	GGGGGGGGGGGGGGG (SEQ ID NO:13)
	BSAP	GACGCANYGRWNNNMG (SEQ ID NO:14)
	CBF	ACACCCAAATATGGCGAC (SEQ ID NO:15)
20	C/EBP	GTGGWWWG (SEQ ID NO:16)
	CF1	ANATGG (SEQ ID NO:17)
	COUP	GTGTCAAAGGTCA (SEQ ID NO:18)
	CP1	YNNNNNNRRCCAATCANYK (SEQ ID NO:19)
	CP2	YAGYNNNRCCAATCNNNR (SEQ ID NO:20)
25	CTCF	CCCTC (SEQ ID NO:21)
	DBP	TGATTTTGT (SEQ ID NO:22)
	E2A	RCAGNTG (SEQ ID NO:23)
	E2B	TGCAAYAY (SEQ ID NO:24)
	E2F	TTTTSSCGS (SEQ ID NO:25)
30	E4F	TGACGTAAC (SEQ ID NO:26)
	EGR-1	CGCCSCGC (SEQ ID NO:27)
	EGR-2	CCGCCCCGC (SEQ ID NO:28)
	ER	AGGTCANNNTGACCT (SEQ ID NO:29)
	v-ErbA	GTGTCAAAGGTCA (SEQ ID NO:30)

	ETF	CAGCCCCGCGCAGC (SEQ ID NO:31)
	Ets-1	SMGGAWGY (SEQ ID NO:32)
	F-ACT1	TGGCGA (SEQ ID NO:33)
	GAL 4	CGGN <sub>5</sub> (T/A)N <sub>5</sub> CCG (SEQ ID NO:34)
5	GATA-1	WGATAR (SEQ ID NO:35)
	GATA-2	WGATAR (SEQ ID NO:36)
	GATA-3	WGATAR (SEQ ID NO:37)
	GCF	SCGSSSC (SEQ ID NO:38)
	GHF-1	WTATYCAT (SEQ ID NO:39)
10	GHF-5	WTATYCAT (SEQ ID NO:40)
	GHF-7	WTATYCAT (SEQ ID NO:41)
	GR	AGAACANNNTGTTCT (SEQ ID NO:42)
	H1TF2	GCACCAATCACAGCGCGC (SEQ ID NO:43)
	H2RIIBP	TCAGGTCACAGTGACCTGA (SEQ ID NO:44)
15	H2TF1	TGGGGATTCCCCA (SEQ ID NO:45)
	H-APF-1	CTGGRAA (SEQ ID NO:46)
	HIF	CTACGTGCT (SEQ ID NO:47)
	HNF-1	GTTAATNATTAAC (SEQ ID NO:48)
	vHNF-1	GTTAATNATTAAC (SEQ ID NO:49)
20	HNF-3A	TATTGAYTTWG (SEQ ID NO:50)
	HNF-3B	TATTGAYTTWG (SEQ ID NO:51)
	HNF-3C	TATTGAYTTWG (SEQ ID NO:52)
	HNF-4	KGCWARGKYCAY (SEQ ID NO:53)
	HSF	NGAANNGAANNGAAN (SEQ ID NO:54)
25	IAF	GCCATCTGCT (SEQ ID NO:55)
	IRBP	AGTGCACT (SEQ ID NO:56)
	IREBF-1	CGGGAATGGAACTG (SEQ ID NO:57)
	IRF	AANNGA (SEQ ID NO:58)
	ISGF1	CTTTCAGTTT (SEQ ID NO:59)
30	ISGF2	CTTTCCTTTT (SEQ ID NO:60)
	ISGF3	GCTTCAGTTT (SEQ ID NO:61)
	KBF-1	TGGGGATTCCCCA (SEQ ID NO:62)
	Ker1	GCCTGCAGGC (SEQ ID NO:63)
	LFB3	GTTAATNATTAAC (SEQ ID NO:64)



	LIT-1	GCGCCCTTTGGACCT (SEQ ID NO:65)
	LyF-1	PPTGGGAGR (SEQ ID NO:66)
	MBF-1	YTAAAAATAAYYY (SEQ ID NO:67)
	MBF-I	TGCRRC (SEQ ID NO:68)
5	MBP-1	TGGGGATTCCCA (SEQ ID NO:69)
	MCBF	CATTCCT (SEQ ID NO:70)
	MEF-2	YTAWAAATAR (SEQ ID NO:71)
	MEP-1	TGCRNC (SEQ ID NO:72)
	MR	AGAACANNNITGTCT (SEQ ID NO:73)
10	Myb	YAACKG (SEQ ID NO:74)
	Myc	CACGTG (SEQ ID NO:75)
		TCTCTTA
	MycD	CAACTGAC (SEQ ID NO:76)
	NF1	YGGMNNNNNGCCAA (SEQ ID NO:77)
15	NF-AT	GGAGGAAAACTGTTTCAT (SEQ ID NO:78)
	NF-E2	TGACTCAG (SEQ ID NO:79)
	NF-D	GATGGCGG (SEQ ID NO:80)
	NF-GMa	GRGRGTTKAY (SEQ ID NO:81)
	NF-GMb	TCAGRTA (SEQ ID NO:82)
20	NF-IL6	TKNNGNAAK (SEQ ID NO:83)
	NFxB	GGGAMTNYCC (SEQ ID NO:84)
	NF-W1	GTTGCATC (SEQ ID NO:85)
	NF-W2	GTTGCATC (SEQ ID NO:86)
	NGF1-B	AGGTCATGACCT (SEQ ID NO:87)
25	Oct-1	ATGCAAAT (SEQ ID NO:88)
	Oct-2	ATGCAAAT (SEQ ID NO:89)
	Oct-4	ATGCWAAT (SEQ ID NO:90)
	Oct-6	ATGCAAAT (SEQ ID NO:90)
	P53	RRRC(A/T) (T/A) GYYY (N) <sub>0-13</sub> RRRC(A/T) (T/A) GYYY
30		(SEQ ID NO:92)
	Pax-1	CACCGTTCCGCTCTAGATATCTC (SEQ ID NO:93)
	PCF	AGAAAGGGAAAGGA (SEQ ID NO:94)
	PEA3	AGGAAR (SEQ ID NO:95)
	PPAR	AGGTCA (SEQ ID NO:96)

	PR	AGAACANNNTGTTCT (SEQ ID NO:97)
	PRDI-BF1	AAGTGAAAGT (SEQ ID NO:98)
	PTF1	ATGGGANCTCAGCTGTGC (SEQ ID NO:99)
	Pu.I	AGAGGAACT (SEQ ID NO:100)
5	PuF	GGGTGGG (SEQ ID NO:101)
	RAR	AGGTCATGACCT (SEQ ID NO:102)
	RFX	CCCCTAGCAACAGATG (SEQ ID NO:103)
	Runt	YGYGGT (SEQ ID NO:104)
	RVF	AAGATAAAACC (SEQ ID NO:105)
10	RXR	AGGTCA (in a Direct Repeat configuration) (SEQ ID NO:106)
	SIF	CCCGTM (SEQ ID NO:107)
	Sp1	KRGGCTRRK (SEQ ID NO:108)
	SREBP1	ATCACGTGA (E-Box consensus binding sequence) (SEQ ID NO:109)
15		or ATCACCCAC (non E-Box consensus bindingsequence) (SEQ ID NO:110)
	SRF	GGATGTCCATATTAGGACATCT (SEQ ID NO:111)
	STAT	TTCNNNGAA (SEQ ID NO:112)
20	T3R	AGGTCAATGACCT (SEQ ID NO:113)
	TBP	TATAAA (SEQ ID NO:114)
	TCF-1	MAMAG (SEQ ID NO:115)
	TCF-2.alpha.	SAGGAAGY (SEQ ID NO:116)
	TEF-1	AAGYATGCA (SEQ ID NO:117)
25	TEF-2	GGGTGTGG (SEQ ID NO:118)
	TGT3	AAGTGTITGC (SEQ ID NO:119)
	TIN-1	AGGAAGTCC (SEQ ID NO:120)
	USF	CACGTG (SEQ ID NO:121)
	WT-ZFP	CGCCCCCGC (SEQ ID NO:122)
30	XF1/2	TCTTCTCAGCAACT (SEQ ID NO:123)
	XPF-1	CACCTGNNNNTTTCCC (SEQ ID NO:124)
	YB-1	ATTTTCTGATTGGCCAAAG (SEQ ID NO:125)

List of viral proteins binding to DNA

- Epstein-Barr  
GGT TAG CAT ATG CTA ACC A (SEQ ID NO:126)
- 5 Virus EBNA (SEQ ID NO:127)  
(B958 strain)  
Epstein-Barr  
T TAG CAA TG (SEQ ID NO:128)
- Virus BZLF  
10 (B958 strain)  
Human CBF-1 CGTGGGAA (EpsteinBarr Virus cis-element)  
(SEQ ID NO:129)  
Human Papilloma A CCG AAA ACG GTG T (SEQ ID NO:130)  
Herpes Simplex
- 15 ATG CTA ATG ATA (SEQ ID NO:132)  
Virus Type 1 VP16  
HIV TAT GGG TCT CTC TGG TTA GAC CAG  
ATC TGA GCC TGG GAG CTC TCT  
GGC TAA CTA GGG AAC CCA
- 20 (SEQ ID NO:133)  
(TAR RNA SEQUENCE)  
HIV Integrase GTGTGGAAAATCTCTAGCA (SEQ ID NO:134)
- Abbreviations : R = (A,g) - Y = (C,T) - M = (A,C) - K =  
25 (g,T) - S = (g,C) - H = (A,T,C) - B = (g,T,C) - D = (g,A,T)  
- N = (A,g,C,T) - V = (g,A,C) - X = (C,I) - W = (A,T) - O =  
Sme-dC - Q = 5Br-dU - I = Inosine

CLAIMS

1. A screening and/or quantification method of one or more transcriptional factor(s) (1) present in a cell or cell lysate, said method comprising the steps of:
- 5 - binding to an insoluble solid support (3), double-stranded DNA sequence(s) (2) at the concentration of at least  $0.01 \text{ pmole/cm}^2$  of said solid support surface, said double-stranded DNA sequence comprising a specific sequence able to bind said transcriptional factor (1),
- 10 - putting into contact said transcriptional factor (1) with said bound double-stranded DNA sequence(s) (2), and
- identifying and/or quantifying a signal resulting from the binding of said transcriptional factor(s) (1) upon said double-stranded DNA sequence(s) (2).
- 15 2. The method according to claim 1, wherein the transcriptional factor is present in solution at concentration lower than 20 nmolar (nM).
3. The method according to claim 1, wherein the specific sequence of the double-stranded DNA
- 20 sequence(s) (2) able to bind with the transcriptional factor(s) (1) is located at a distance of at least about 6,8 nm from the surface of the solid support (3).
4. The method according to claim 1, wherein the signal resulting from the binding of the
- 25 transcriptional factor (1) upon the double-stranded DNA sequence (2) is a non radioactive resulting signal.
5. The method according to claim 1, wherein the signal resulting from the binding of the transcriptional factor (1) upon the double-stranded DNA
- 30 sequence (2) is obtained through an enzymatic reaction
6. The method according to any of the preceding claims 1 to 3, for the (possibly simultaneous) screening and/or quantification of multiple different

transcriptional factors (1) present in a same biological sample.

7. The method according to any of the preceding claims 1 to 6, for the screening and/or  
5 quantification of transcriptional factors (1) selected from the group consisting of NF-KB, AP-1, CREB, SP-1, C/EBP, GR, HIF-1, Myc, NF-AT, Oct, TBP and CBF-1 or factors listed in table 1.

8. The method according to any of the  
10 preceding claims 1 to 7, for the screening and/or quantification of multiple different transcriptional factors (1) upon a same support preferably upon the same multiwell plate.

9. The method according to any of the  
15 preceding claims 1 to 8, wherein the solid support (3) is an array bearing upon at least 4 spots/cm<sup>2</sup> of solid support surface, each spot containing double-stranded DNA sequence(s) (2) for the binding of transcriptional factor(s) (1).

20 10. The method according to any one of the preceding claims 1 to 9, wherein the double-stranded DNA sequence(s) (2) comprise(s), between the specific sequence able to bind the transcriptional factor(s) (1) and the solid support (3), a spacer of at least about 13.5 nm.

25 11. The method according to claim 10, wherein said spacer is a double-stranded DNA nucleotide sequence of at least 20 base pairs, preferably at least 40 base pairs.

12. The method according to any one of the  
30 preceding claims 1 to 11, wherein the double-stranded DNA sequence(s) (2) are bound to a first member (6) of a binding pair (preferably biotin) able to interact with a second member (7) of said binding pair (preferably

streptavidin) bound to the surface of the solid support (3).

13. The method according to any one of the preceding claims 1 to 12, wherein the double-stranded DNA  
5 sequence(s) (2) are covalently bound to the surface of the insoluble solid support (3).

14. The method according to any one of the preceding claims 1 to 13, wherein the consensus sequence is repeated on the same molecule.

10 15. The method according to any of the preceding claims 1 to 14, wherein the double-stranded DNA sequences fixed on the support surface contain in part or totally one or several of the consensus DNA sequences presented in the table 1.

16. The method according to any of the preceding claims 1 to 15, wherein said transcriptional factor is the HIV integrase.

17. The method according to any one of the preceding claims 1 to 16, comprising the step of  
20 identification of at least one characteristic specific of the transcriptional factor activation.

18. The method according to any of the preceding claims 1 to 16, which comprises the steps of screening, quantifying and/or recovering compounds able to  
25 bind to said transcriptional factor(s) or inhibit the binding of transcriptional factor(s) (1) to the specific sequence upon the double-stranded DNA sequence(s) (2) bound to said solid support (3).

19. The method according to any of the preceding claims 1 to 16, which comprises the steps of  
30 screening, quantifying and/or recovering compounds which modulate the binding and/or the activity of the said transcriptional factor(s) when they are put in contact with cells, tissues or organisms.

20. The method according to any of the preceding claims 1 to 16, which comprises the steps of screening, quantifying and/or recovering compounds which modulate the activity of enzyme(s) or protein(s) acting on  
5 transcriptional factor(s) and then assayed for the binding to and/or activity of said transcriptional factor(s).

21. A method according to any of the preceding claims 1 to 16, which comprises the step of identification of transcriptional factor(s) and/or of  
10 peptides which are part of their active complex.

22. The method according to any one of the preceding claims 1 to 16, which comprises the step of adding in the cell lysate an externally added transcriptional factor or a compound which is able to bind  
15 to the consensus sequence.

23. A screening, diagnostic and/or quantification kit comprising means and media for performing the method according to any one of the preceding claims 1 to 22.

20 24. The kit according to claim 23 for the screening and/or quantification of a transcriptional factor(s) (1) or a compound able to bind to said transcriptional factor(s) (1) or inhibit the binding of said transcriptional factor(s) (1) to a specific nucleotide  
25 sequence, which comprises double-stranded DNA sequence(s) (3) bound to an insoluble solid support (3) at a concentration of at least about 0.01 pmole/cm<sup>2</sup> of solid support surface (3).

25 25. The kit according to the claim 23 or 24, comprising a solid support bearing on its surface one or several double stranded DNA consensus sequences at a concentration of at least 0.01 pmole/cm<sup>2</sup> comprising in part or totally one or several of the consensus sequence(s)

listed in table 1 allowing the binding of transcriptional factor(s) present in solution and their detection and/or quantification.

26. The kit according to any of the  
5 preceding claims 23 to 25, wherein the solid support is an array having at least 4 spot/cm<sup>2</sup> of solid support surface containing double-stranded DNA sequence(s) (2) for the binding of the transcriptional factor(s) (1).

27. The kit according to any of the  
10 preceding claims 23 to 26, wherein the double-stranded DNA sequence(s) (2) comprises between the specific sequence able to bind the transcriptional factor(s) (1) and the surface of the solid support (3), a spacer of at least about 13.5 nm.

28. The kit according to any of the  
15 preceding claims 23 to 27, wherein said spacer is a double-stranded DNA nucleotide sequence of at least 20 base pairs.

29. The kit according to the claim 27,  
20 wherein the spacer is a double-stranded DNA nucleotide sequence of at least 40 base pairs.

30. The kit according to any of the  
preceding claims 23 to 29, wherein the double-stranded DNA sequence(s) (2) are bound to a first member (6) of a  
25 binding pair (preferably biotin), able to interact with a second member (7) of the binding pair (preferably streptavidin) bound to the surface of the solid support (3).

31. The kit according to any of the  
30 preceding claims 23 to 29, wherein the double-stranded DNA sequence(s) (2) are covalently bound to the surface of the solid support.



32. Use of the screening and/or quantification kit according to any of the preceding claims 23 to 31, for the screening and/or the quantification of a transcriptional factor(s) present in a cell or a cell lysate.

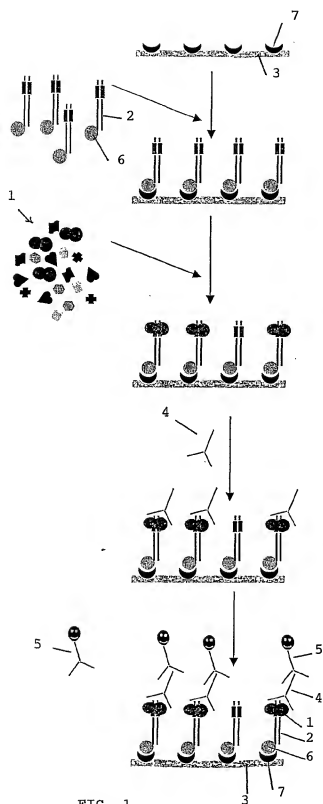
33. The use of the screening and/or quantification kit according to any of the preceding claims 23 to 31, for the screening, the identification, the detection and/or the quantification of a compound able to bind to a transcriptional factor(s) or able to inhibit the binding of a transcriptional factor(s) to a specific nucleotide sequence.

34. The use of the screening and/or quantification kit according to any of the preceding claims 23 to 31, for the screening, the identification, the detection and/or the quantification of a compound able to activate or inactivate a transcriptional factor(s).

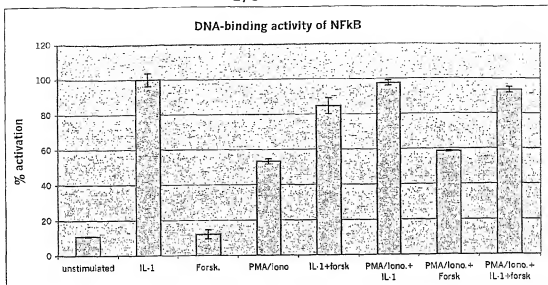
35. The use according to one of the preceding claims 32 to 34, wherein said transcriptional factor(s) are selected from the group consisting of NF-KB, AP-1, CREB, SP-1, C/EBP, GR, HIF-1, Myc, NF-AT, Oct, TBP and CBF-1 or factors listed in table 1.

36. Unknown compound identified and/or recovered by the method according to the claim 19 to 21.

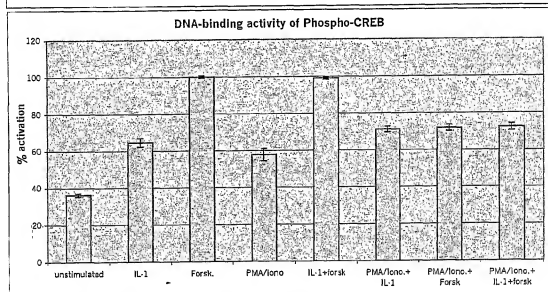
37. Pharmaceutical composition comprising an adequate pharmaceutical carrier and the unknown compound according to claim 36.



A.



B.



C.

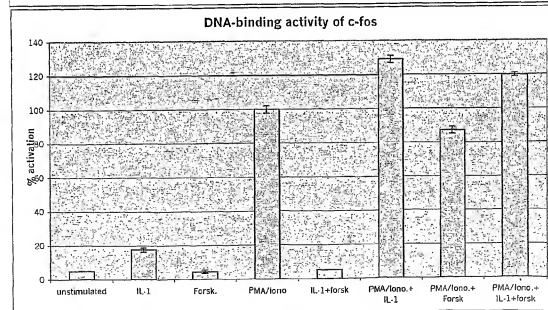
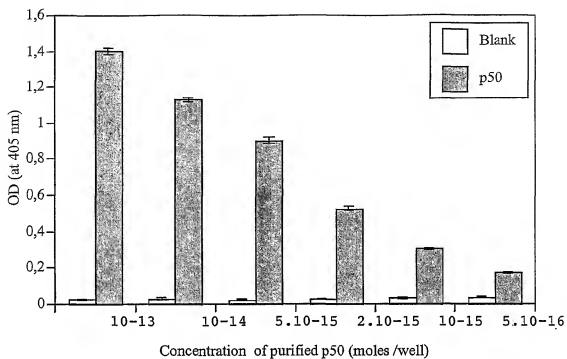
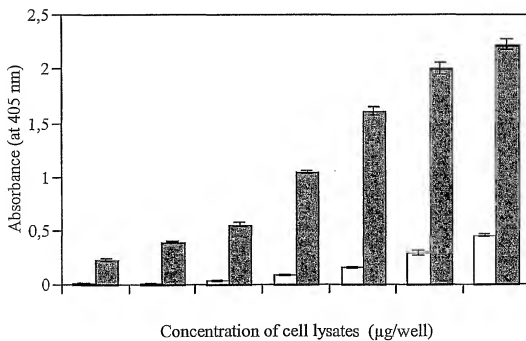
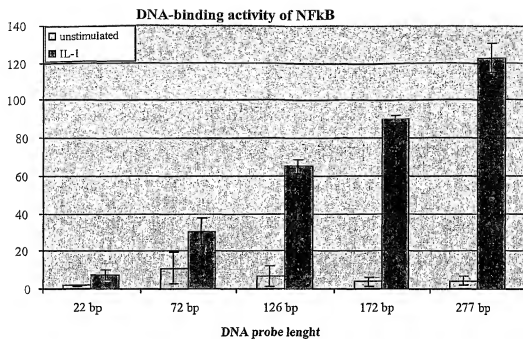


FIG. 2

3/5

FIG. 3FIG. 4

4/5

FIG. 5

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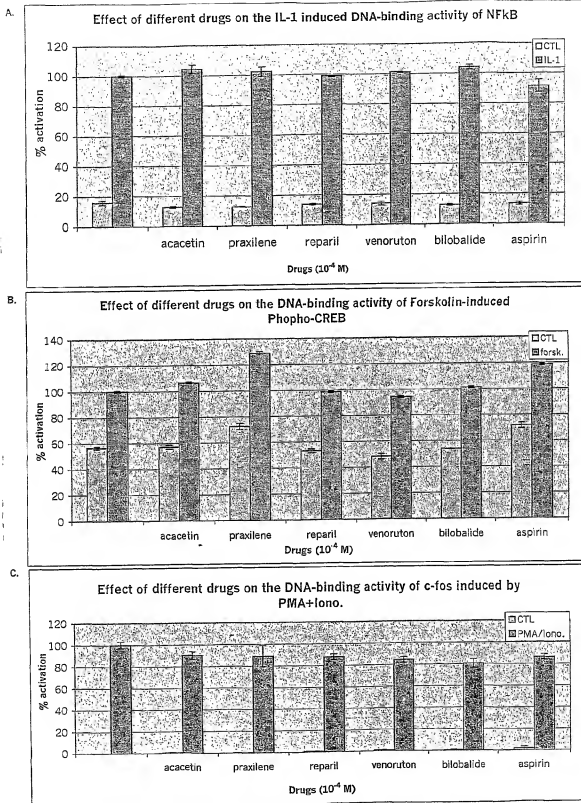


FIG. 6

## INTERNATIONAL SEARCH REPORT

ational Application No.

/BE 01/00052

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 C12Q1/68 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIBMA M H ET AL: "A NON-RADIOACTIVE ASSAY FOR THE DETECTION AND QUANTITATION OF A DNA BINDING PROTEIN" NUCLEIC ACIDS RESEARCH, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 22, no. 18, 11 September 1994 (1994-09-11), pages 3806-3807, XP000673300 ISSN: 0305-1048	1-15, 17-35
Y	the whole document	16
Y	WO 95 30026 A (TULARIK INC) 9 November 1995 (1995-11-09) the whole document	1-35
Y	EP 0 620 439 A (BOEHRINGER MANNHEIM GMBH) 19 October 1994 (1994-10-19) the whole document	1-35
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

17 August 2001

Date of mailing of the international search report

23/08/2001

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## INTERNATIONAL SEARCH REPORT

International Application No.

P/BE 01/00052

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 08096 A (AMSTERDAM SUPPORT DIAGNOSTICS ;Goudsmit Jaap (NL); Wolf Frank de ( ) 26 February 1998 (1998-02-26) the whole document ---	1-35
Y	Mazumder A et al: "PROBING INTERACTIONS BETWEEN VIRAL DNA AND HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INTEGRASE USING DINUCLEOTIDES" MOLECULAR PHARMACOLOGY, BALTIMORE, MD, US, vol. 51, no. 4, April 1997 (1997-04), pages 567-575, XP002920201 ISSN: 0026-895X the whole document ---	16
A	WO 89 11548 A (CETUS CORP) 30 November 1989 (1989-11-30) the whole document ---	
A	Guo Z et al: "DIRECT FLUORESCENCE ANALYSIS OF GENETIC POLYMORPHISMS BY HYBRIDIZATION WITH OLIGONUCLEOTIDE ARRAYS ON GLASS SUPPORTS" NUCLEIC ACIDS RESEARCH, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 22, no. 24, 11 December 1994 (1994-12-11), pages 5456-5465, XP002006248 ISSN: 0305-1048 the whole document ---	
A	Zammatteo Nathalie et al: "Comparison between microwell and bead supports for the detection of human cytomegalovirus amplicons by sandwich hybridization." ANALYTICAL BIOCHEMISTRY, vol. 253, no. 2, 15 November 1997 (1997-11-15), pages 180-189, XP000939345 ISSN: 0003-2697 the whole document -----	



## FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Continuation of Box I.2

Claims Nos.: 36,37

Present claims 36 and 37 relate to a compound defined by reference to a desirable characteristic or property, namely by its ability to modulate the activity of a transcriptional factor. The claims cover all compounds having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search of these claims is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search of the claims impossible. Consequently, no search has been carried out for claims 36 and 37.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PC/BE 01/00052

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9530026 A	09-11-1995	US 5563036 A	08-10-1996
		AU 679375 B	26-06-1997
		AU 2368195 A	29-11-1995
		CA 2163658 A	09-11-1995
		EP 0715656 A	12-06-1996
		JP 2953783 B	27-09-1999
		JP 8507222 T	06-08-1996
EP 0620439 A	19-10-1994	DE 4312399 A	20-10-1994
WO 9808096 A	26-02-1998	NL 1003839 C	26-02-1998
		AU 3953997 A	06-03-1998
WO 8911548 A	30-11-1989	AT 173508 T	15-12-1998
		AU 632494 B	07-01-1993
		AU 3754289 A	12-12-1989
		DE 68928853 D	24-12-1998
		DE 68928853 T	05-08-1999
		EP 0451141 A	16-10-1991
		IE 164389 L	20-11-1989
		IL 90358 A	18-08-1993
		JP 2897959 B	31-05-1999
		JP 3504328 T	26-09-1991